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Natural Catalytic Antibodies: Peptide-hydrolyzing Activities of Bence Jones Proteins and V_L Fragment*

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Monoclonal human light chains, *i.e.* Bence Jones proteins, and their recombinant variable fragments (V_L) were screened for proteolytic activity using peptide-methylcoumarinamide (peptide-MCA) conjugates and vasoactive intestinal polypeptide (VIP) as substrates. Sixteen of 21 Bence Jones proteins and one of three V_L fragments were capable of detectable cleavage of one or more substrates. The magnitude and kinetic characteristics of the activity varied with different substrates. Among the peptide-MCA substrates, the presence of tripeptide or tetrapeptide moieties with a basic residue at the scissile bond generally favored expression of the activity. The influence of N-terminal flanking residue recognition was evident from differing values of K_m and k_{cat} (turnover number) observed using different Arg-containing peptide-MCA substrates. Different light chains displayed different kinetic parameters for the same substrate, suggesting unique catalytic sites. Hydrolysis of VIP was characterized by nanomolar Michaelis-Menten constants (K_m), suggesting comparatively high affinity recognition of this peptide. The 25-kDa monomer and the 50-kDa dimer forms of one light chain preparation were resolved by gel filtration in 6 M guanidine hydrochloride. Following renaturation, the monomer displayed 51-fold greater peptide-MCA-hydrolyzing activity than the dimer. A renatured V_L domain prepared by gel filtration in 6 M guanidine hydrochloride displayed VIP-hydrolyzing activity in the 12.5-kDa peak fractions. These results provide evidence for the proteolytic activity of certain human light chains and imply that this phenomenon may have a pathophysiological significance.

Bence Jones proteins are monoclonal antibody light chains found in the urine of approximately 60% of multiple myeloma patients (1, 2). The mechanisms underlying the pathophysiology of these tumors are not understood fully. Light chain aggregation has been advanced as one of the causes of tissue damage, particularly involving the kidney (3). The antibodies produced by tumor cells in multiple myeloma are described to bind certain antigens, including determinants found in carbohydrates (4) and autoantigens expressed by neuronal cells (5, 6). These findings have raised the possibility that antigen recognition by the tumor cell antibody products may contribute to the pathophysiology of multiple myeloma.

Some antibodies possess catalytic activities similar to those of enzymes (7–10). Antibody light chain subunits free of heavy chains can bind antigen (11, 12). The catalytic activity of one of these antibodies has been localized to an active site in the light chain subunit (13, 14). Bence Jones proteins accumulate to millimolar levels in multiple myeloma patients (15, 16), at which even low level catalytic activity may be biologically important. Here, we describe evidence for peptidase activity attributable to the variable domain in a majority of light chains isolated from myeloma patients. This observation indicates that catalysis by antibody subunits is not a rare phenomenon and suggests a possible link between antibody catalysis and the pathophysiology of multiple myeloma.

MATERIALS AND METHODS

Bence Jones Proteins and V_L Fragments—Monoclonal κ and λ light chains were purified from the urine of 21 myeloma patients with multiple myeloma as described previously (17). Recombinant V_L domains (rREC, rLEN, rREI) were expressed in *Escherichia coli* from synthetic genes and purified as in Ref. 18. SDS-polyacrylamide gel electrophoreses in the absence or presence of 20 mM 2-mercaptoethanol was on Phast gradient gels (8–25%; Pharmacia Biotech Inc.). Each of the 21 light chain preparations contained a 50-kDa dimer band and a 25-kDa monomer band (Fig. 1). The disulfide bond in the dimer was reduced in mercaptoethanol, producing a single 25-kDa monomer band. A single 12.5 kDa band was observed in the two V_L preparations. Gel filtration of light chain and V_L was in 6 M guanidine hydrochloride, pH 6.5 (Sigma) on a Superose-12 fast protein liquid chromatography column (Pharmacia). The column fractions were renatured by dialysis against 50 mM Tris-HCl, 100 mM glycine, 0.025% Tween 20, 0.02% sodium azide, pH 7.7, for 2 days with four buffer changes using a Life Technologies, Inc. multiwell dialysis device (final guanidine concentration < 1 nM, assuming equilibration across the dialysis membrane).

Catalysis Assays—Light chains were incubated with peptide-MCA[1] conjugates (Peptides International or Sigma) in 50 μl of 50 mM Tris-HCl, 100 mM glycine, 0.025% Tween 20, pH 7.7, in 96-well plates (MicroFluor W, Dynatech) at 37 °C in a humidified incubator. Hydrolysis of peptide-MCA substrates was determined as the fluorescence of the aminomethylcoumarin leaving group (λ_{em} 460 nm, λ_{ex} 370 nm) using a plate reader (Perkin Elmer LS50 fluorimeter). Product concentrations were computed by comparison of the fluorescence yield with aminomethylcoumarin (Peptides International) measured in identical volumes (18.3 fluorescence units/μM/50 μl). Background fluorescence measured in wells containing the substrate in diluent was generally less than 20 fluorescence units and was subtracted from values observed in the presence of catalyst. [Tyr^{10}-^{125}I]VIP prepared as in (19) was incubated with light chains at 37 °C in 200 μl of 50 mM Tris-HCl, 100 mM glycine, 0.025% Tween 20, pH 7.7, for 6 h, and peptide hydrol-

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[1] The abbreviations used are: MCA, methylcoumarinamide; VIP, vasoactive intestinal polypeptide; V_L, variable domain of light chain; Boc, *t*-butyloxycarbonyl.

ysis was estimated by measuring the radioactivity soluble in 10% trichloroacetic acid. Estimates of peptide breakdown by this method are essentially identical to those obtained by reversed-phase high performance liquid chromatography separation of intact and degraded fragments (20). Trypsin was from bovine pancreas (3× crystallized, 3080 units/mg; U. S. Biochemical Corp). Initial rate data at varying substrate concentration were fitted using nonlinear regression analysis to the Michaelis-Menten-Henri equation ($v = (V_{max}[S])/(K_m + [S])$) (Enzfitter, Elsevier-Biosoft). Since the catalyst concentration in some assays was in the range of the low end of VIP substrate concentrations analyzed, kinetic constants were also computed by a graphing routine as described in Ref. 21, which eliminates errors due to substrate depletion effects. The K_s and k_{cat} values estimated by this method were essentially identical (<10% difference) to the kinetic constants reported (see Table III).

RESULTS

VIP and Peptide-MCA Cleavage—Twenty-one myeloma light chains and three V_L domains were initially screened for proteolytic activity using radiodiodinated VIP as substrate, a 28-amino acid neuropeptide previously shown to serve as a target for catalytic antibodies (7, 14). Four light chains and one recombinant V_L preparation hydrolyzed radiolabeled VIP (Table I). Further screening was performed using a panel of five peptide-MCA conjugates. Cleavage of the amide bond linking an amino acid with the terminal coumarin moiety in these conjugates serves as a surrogate for peptide bond hydrolysis, permitting rapid measurement of protease activity (22). Arg-MCA- and Lys-MCA-containing substrates were employed in the screening assays because recombinant anti-VIP light chains, thyroglobulin-specific autoantibodies, and polyclonal IgG preparations have previously been shown to display pronounced preference for cleavage at bonds containing basic amino acids (10, 14). A majority of the light chains (76%) and one V_L preparation displayed detectable hydrolytic activity with one or more peptide-MCA substrates (Table I). Wide variations were evident in the levels of hydrolysis of different substrates by individual light chains and of individual substrates by different light chains. The greatest frequency of hydrolysis was observed with Boc-Glu-Ala-Arg-MCA as substrate. Four light chain preparations displayed detectable hydrolysis of all five peptide-MCA substrates, and two displayed hydrolysis of four substrates.

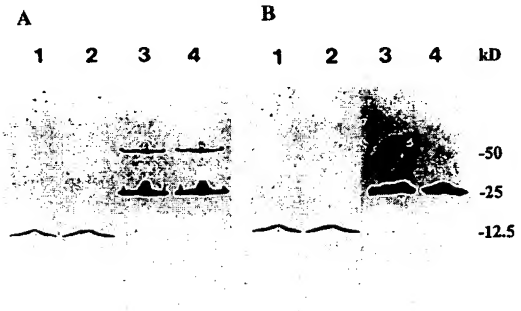

FIG. 1. **Silver-stained SDS-polyacrylamide gels run in nonreducing (*A*) and reducing (*B*) conditions.** *Lanes 1* and *2*, recombinant V_L fragments rRec and rLEN, respectively. *Lanes 3* and *4*, Bence Jones proteins B6 and RHY, respectively.

Cleavage of 10 additional MCA-conjugates of varying lengths and containing uncharged, acidic, or aromatic residues at the scissile bond by two light chains was measured (Table II). The activity of one of these light chains (Bence Jones protein B6) was comparatively indiscriminate, in that there was detectable cleavage at Ala-MCA, Phe-MCA, Met-MCA, Arg-MCA, and Lys-MCA bonds. Even single amino acid-MCA conjugates were cleaved by this light chain, but with increasing chain length, the activity acquired preference for Arg- and Lys-containing bonds. The second light chain (Bence Jones protein RHY) displayed essentially no hydrolysis of substrates containing MCA linked to uncharged, acidic, or aromatic amino acids. These observations indicate preferential cleavage at basic residue-MCA bonds by the light chains and a variable influence of N-terminal flanking residues in catalysis by the two light chains.

Kinetics—The initial rate data obtained at increasing substrate concentrations were fitted to the Michaelis-Menten equation (Fig. 2). The K_m for light chain-catalyzed hydrolysis

TABLE I

Peptidase activity of Bence Jones protein and recombinant V_L fragment

Values are ΔF/20 h for peptide-MCA substrates and percent hydrolysis/6 h for ^{125}I-VIP. ND denotes not detectable (ΔF for peptide-MCA substrates < 11, percent VIP hydrolysis < 10). Light chain isotype and subgroup is given in parentheses. Peptide-MCA and VIP substrate concentrations, 0.2 mM and 0.5 nM, respectively. Catalyst concentrations in peptide-MCA and VIP hydrolysis assays, 2 μM and 0.25 μM, respectively. rREI, rLEN, rREC are recombinant V_L fragments. The remaining proteins are light chains purified from urine. Amino acids are identified by one-letter codes.

Protein i.d.	PFR-MCA	Boc-IEGR-MCA	Boc-EAR-MCA	Boc-VLK-MCA	EKK-MCA	^{125}I-VIP
B6 (κIII)	58	366	878	31	49	12
BOR (κI)	ND	ND	11	11	ND	ND
BOS (λII)	ND	ND	26	ND	ND	42
COL (κI)	ND	ND	ND	ND	ND	ND
CRO (κI)	12	45	52	34	40	45
EVE (λII)	ND	ND	27	ND	14	ND
FRE (λIII)	ND	ND	35	13	ND	ND
HAS (λIII)	ND	ND	32	12	ND	ND
HOU (κI)	ND	ND	49	18	16	ND
JOH (κI)	69	318	213	51	112	ND
LAYM (κII)	ND	72	358	20	15	79
LEN (κIV)	ND	ND	ND	ND	ND	ND
LOC (λI)	15	34	194	ND	ND	ND
MCP (λII)	ND	ND	14	ND	ND	ND
MOR (λVI)	ND	ND	ND	ND	ND	ND
RHY (κIII)	58	811	783	186	158	ND
ROB (κIII)	ND	ND	ND	ND	ND	ND
UND (κI)	ND	59	81	18	13	ND
XOC (κIII)	ND	ND	ND	ND	ND	ND
WAT (κI)	ND	ND	18	ND	ND	ND
WIT (λIII)	ND	ND	16	ND	ND	ND
rREI (κI)	ND	ND	ND	ND	ND	ND
rLEN (κIV)	ND	ND	ND	ND	ND	ND
rREC (κIV)	20	21	ND	19	15	61

TABLE II

Cleavage of various peptide-methylcoumarinamide substrates by two Bence Jones proteins, B6 and RHY

Values are ΔF/20 h from a representative experiment performed at 200 μM substrate and 2 μM light chain concentrations.

Substrate	B6	RHY
K-MCA	47	1
R-MCA	57	1
A-MCA	106	0
F-MCA	28	1
KA-MCA	2	1
AE-MCA	0	3
FR-MCA	12	33
AAA-MCA	0	2
VLM-MCA	15	0
AAPF-MCA	1	0
LLVY-MCA	0	0
PFR-MCA	58	58
Boc-IEGR-MCA	366	811
Boc-EAR-MCA	878	783
Boc-VLK-MCA	31	186
E-KK-MCA	49	158

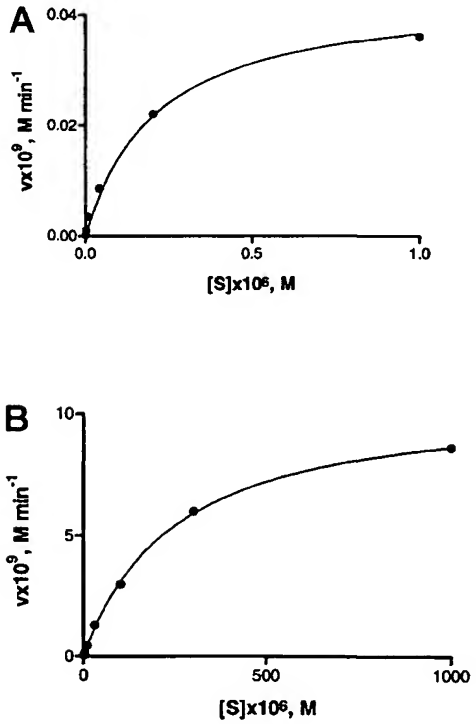

FIG. 2. **Initial rate data for VIP hydrolysis by rREC V_L fragment (*A*) and Boc-Ile-Glu-Gly-Arg-MCA hydrolysis by Bence Jones protein B6 (*B*) fitted to the Michaelis-Menten equation.** Catalyst concentrations: B6, 125 nM; rREC, 25 nM. Values are means of closely agreeing duplicate determinations. Reaction times were 20 h and 6 h for Boc-Ile-Glu-Gly-Arg-MCA and VIP, respectively.

for the peptide-MCA substrates ranged from 15–290 μM (Table III), values in the same range as those observed for conventional proteases. In contrast to the peptide-MCA substrates, VIP hydrolysis by both catalyst preparations examined was notable for low K_m values, suggesting comparatively high affinity recognition of this substrate. In comparison, the K_m for trypsin-catalyzed VIP hydrolysis, estimated by methods identical to those employed here, is approximately 3 orders of magnitude greater (14). Because of the low K_m values, the kinetic efficiencies (k_{cat}/K_m) of the VIP-hydrolyzing light chains approach those of conventional proteases. Apparent turnover numbers of the light chain (k_{cat}) are in the same range or higher than of acyltransferase antibodies raised by immunization with presumed transition state analogs (for review, see Ref. 23). The hydrolysis of Boc-Ile-Glu-Gly-Arg-MCA and Boc-Glu-Ala-Arg-MCA by the same light chain (B6) was characterized by kinetic parameters that varied over 1 order of magnitude, confirming the conclusion that residues flanking the scissile bond (P_2, P_3, P_4) play a role in substrate recognition.

TABLE III

Kinetic constants for catalysis by Bence Jones proteins

K_m and k_{cat} values were computed by fitting initial rate data at increasing substrate concentrations (peptide-MCA substrates, 10–3000 μM; VIP, 10–1000 nM) to the Michaelis-Menten equation. Catalyst concentrations: B6, 125 nM; LAY, 125 nM and 21 nM using Boc-EAR-MCA and VIP substrates, respectively; rREC, 25 nM. S.E. values were <15% in all cases.

Catalyst	Substrate	K_m	k_{cat}	k_{cat}/K_m
		M	min^{-1}	$M^{-1}\ min^{-1}$
B6	Boc-IEGR-MCA	2.4×10^{-4}	9.0×10^{-2}	3.5×10^{3}
B6	Boc-EAR-MCA	1.5×10^{-5}	3.3×10^{-2}	2.3×10^{4}
B6	Boc-EKK-MCA	2.9×10^{-4}	2.1×10^{-2}	0.7×10^{3}
LAYM	Boc-EAR-MCA	5.5×10^{-5}	2.2×10^{-2}	4.0×10^{3}
LAYM	VIP	1.4×10^{-7}	1.4×10^{-2}	1.0×10^{5}
rREC	VIP	2.1×10^{-7}	1.0×10^{-3}	4.9×10^{4}

Mapping of Activity by Gel Filtration—Chromatography experiments were performed in a strong denaturant (6 M guanidine hydrochloride) to preclude noncovalent association of adventitious protease contaminants with light chains, and to dissociate noncovalent light chain dimers and higher order aggregates formed at neutral pH in nondenaturing solvents (3). The VIP-hydrolyzing specific activities of fractions spanning the 12.5 kDa optical density peak of a V_L preparation (Fig. 3*A*) were essentially identical (fractions 46–48, 3.6×10^3, 3.8×10^3, and 3.8×10^3 cpm hydrolyzed/μg of protein/6 h), suggesting catalyst homogeneity. Gel filtration of B6 light chain yielded two optical density peaks corresponding to the disulfide-linked dimer and monomer components (Fig. 3*B*). Most of the peptide-MCA activity in the renatured column fractions was associated with the monomer peak. Rechromatography of the monomer and dimer yielded peptide-MCA-hydrolyzing activity peaks that tracked exactly with the optical density peaks (Fig. 3*C*). The specific activities of fractions spanning the width of the dimer and monomer peaks were essentially constant (*upper panel*, fractions 21–23: 29.4, 31.8, and 27.5 units $\Delta F/\mu g$ of protein/20 h, respectively; *lower panel*, fractions 24–26: 1.4×10^3, 1.5×10^3 and 1.5×10^3 units $\Delta F/\mu g$ of protein/20 h, respectively).

DISCUSSION

The results of our studies show that certain human light chains have the capability to cleave synthetic protease substrates and natural peptides. These light chains were judged to be pure on the basis of SDS-polyacrylamide gel electrophoresis in the presence of a reducing agent. The gel filtration experiments furnish direct proof that the light chain itself is responsible for the observed activity since noncovalent complexation of adventitious proteins with the light chains would be precluded in the strong denaturant employed for chromatography. The expression of VIP-hydrolyzing activity in the 12.5 kDa peak of a recombinant V_L fragment shows that the activity is attributable to the variable domain. This conclusion is supported by observations that many but not all light chains possess the activity, and that different light chains show varying activity levels and distinct specificity profiles.

The presence of basic residues in the peptide substrates appears to favor expression of light chain catalytic activity. However, the sequence requirements are not uniformly stringent, and one light chain even hydrolyzed single amino acid-MCA conjugates. Recognition of the MCA conjugates by the

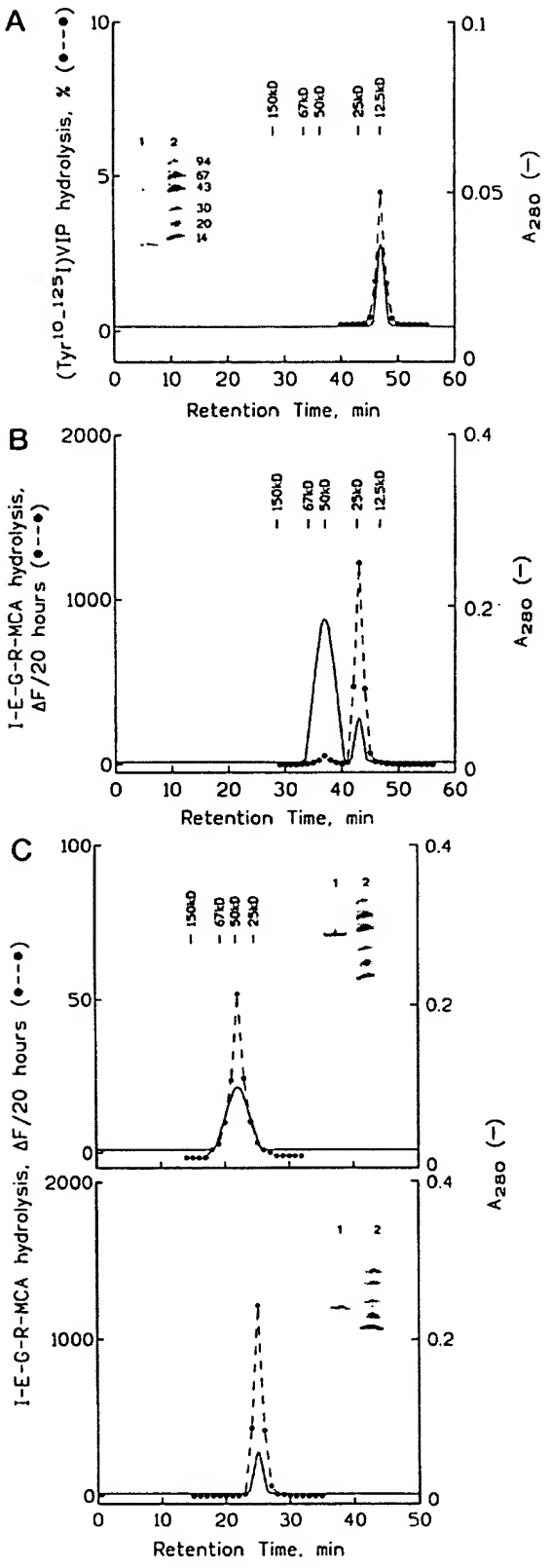

FIG. 3. **Gel filtration of light chain and V_L fragment catalytic activity.** Chromatography was on a Superose-12 gel filtration column in denaturant (6 M guanidine hydrochloride). Effluent fractions were renatured by removal of the guanidine hydrochloride by dialysis. *Insets* are silver-stained SDS-polyacrylamide gels of the peak optical density fraction (*lane 1*) and marker proteins (*lane 2*). *A*, rREC V_L fragment (flow rate, 0.25 ml/min; fraction size, 0.25 ml); *B*, Bence Jones protein B6 (flow rate, 0.25 ml/min; fraction size, 0.25 ml); *C*, rechromatography of B6 dimer (*upper profile*; retention time 37 min in *B*) and B6 monomer (*lower profile*; retention time 43 min in *B*), flow rate 0.5 ml/min, fraction size, 0.5 ml.

light chains does not appear analogous, therefore, to typical high affinity binding interactions between antibodies and polypeptide antigens, which can involve extensive contacts at >15 residues (24). This conclusion is supported by observations of high micromolar K_m values for the peptide-MCA hydrolysis reaction. K_s estimates, obtained as in Ref. 21, are close to the K_m values, suggesting poor binding to the ground state of the substrate. Light chain and V_L fragment catalyzed hydrolyses of VIP, on the other hand, was characterized by lower K_m values, indicating comparatively high affinity recognition of the VIP ground state. VIP binding autoantibodies are found in healthy individuals (25) and patients with asthma (26). The apparent specialization of certain catalytic light chains for binding and hydrolysis of an autologous peptide like VIP is consistent with recognition of other autoantigens by myeloma proteins (4–6). The second feature distinguishing the peptide-MCA substrates and VIP is the frequency with which light chain hydrolytic activity was encountered. Seventeen proteins hydrolyzed the peptide-MCA substrates, whereas only five displayed VIP-hydrolyzing activity. This may reflect differences in structural requirements for cleavage of small *versus* large peptide substrates, as has been described for trypsin (27). For instance, the greater flexibility of small peptides may permit productive contact with the catalytic site, whereas comparatively rigid conformations found in larger molecules may make potential scissile bond inaccessible.

Catalysis has been assumed to be a rare property of antibody subunits. Our finding that 17 of 24 light chains and V_L fragments tested possess detectable proteolytic activity suggests that this may not be an uncommon phenomenon. There is no association evident between the observed catalytic activity and light chain isotype (κ or λ) or V_L subgroup (Table I). Taken together, the high frequency of catalysis and apparent low affinity recognition of structurally different peptide-MCA substrates suggest the presence of conserved structural components of the catalytic site in different light chains. Alternatively, these results may be explained by a tendency toward *de novo* development of catalytic activity during sequence diversification of light chain variable regions. The identities of amino acids responsible for the activity and the contributions of germ line inheritance, V-J gene rearrangement, and somatic mutation mechanisms in catalysis by different light chains are not yet known. A catalytic triad of Ser^{27a}, His^{93}, and Asp^{1} residues (Kabat numbering) in spatial arrangement similar to that of the active site of subtilisin has been identified in an anti-VIP light chain by molecular modeling (14), and site-directed mutagenesis experiments support the role of Ser^{27a} and His^{93} as essential catalytic residues.[2] Erhan and Greller (29) have previously noted a significant sequence similarity between the first complementarity determining region of certain Bence Jones proteins and the active site region in serine proteases.

Light chains dimers serve as models for the antibody combining site (30, 31). In the instance of one light chain studied in detail (B6), the monomer form displayed substantially greater hydrolytic activity than the dimer. Thus, formation of an antibody-like site does not appear to be a prerequisite for catalytically productive substrate contact. This conclusion is relevant to potential intracellular expression of catalytic activity by

[2] Q.-S. Gao, M. Sun, and S. Paul (1995), abstract to be presented at the 9th International Congress of Immunology, San Fransisco, California, July 23–29, 1995.

light chains, wherein a reducing environment is likely to favor the monomer form. The extracellular concentrations of light chains in patients with multiple myeloma can reach millimolar levels (15, 16). Many light chains are capable of cleavage of small protease substrates containing basic residues, and some are capable of cleavage of the larger polypeptide, VIP. These results provide a rationale for examination of light chain-catalyzed peptide breakdown as a pathophysiological mechanism in patients with multiple myeloma. The example of VIP-hydrolysis may be directly relevant to tumor cell activity *in vivo* because this peptide has been shown to regulate antibody and interleukin synthesis by lymphocytes (32–34), and myeloma cells express receptors for VIP (28).

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